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Title

Sebocytes, Sebocyte-cell line and uses thereof

5   **Description**

The present invention relates to grease or lipid containing and
sebum producing cells of the skin and the mucous membrane, also
called sebocytes. The present invention particularly relates to
10   cells of the sebaceous gland and a cell line of sebaceous gland
with the property of being capable to be continuously cultured
through a large number of sub-cultures. The sebocytes are
particularly suitable for useful applications, for example for
the study of the physiology and the pathophysiology of human or
15   animal sebaceous glands, for the study of the generation of
acne, seborrhoe or other diseases, for testing the
effectiveness of various substances and of medicaments, for the
development of cell culture systems being based on two-
dimensional or three-dimensional cell assemblies and
20   constructions of organ-like structures, and for the manufacture
of products being derived from these cells.

Background art

25   Increasing indications suggest that sebocytes play a critical
role in the pathophysiogic processes and diseases of the
sebaceous gland/hair complex, in particular in acne (Gollnick
et al. J. Dermatol. 1991; 18:489-499; Brown and Shalita, Lancet
1998;351:1871-1876; Cunliffe, Dermatology 1998, 196:9-15;
30   Strauss, Dermatology 1998; 196:182-184). The majority of our
understanding of the physiology and pathophysiology of the
sebaceous gland derive from experimental animal models (Pochi
in „Models in Dermatology", Vol. 2, N. Lowe and H. Maibach,
editors, Basel, 1985; 70-75). However, it was found that animal
35   models do not allow reasonable predictions for the evaluation
of the effectiveness of anti-acne medicaments for humans
(Geiger, Dermatology 1995; 1991:305-310). The fact that acne

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occurs only in humans and that the secretion activity of the
sebaceous gland is strongly species specific (Nikkari, J.
Invest. Dermatol. 1974; 257-267) led to the search for human
models. Preliminary studies for the avoidance of these

5   disadvantages was carried out with human skin samples, which
had been either incubated in-vitro (Hsia et al., Proc. Soc.
Exp. Biol. Med. 1970; 135:285-291; Cooper et al., Br. J.
Dermatol. 1976; 94:156-172; Sharp et al., J. Endrocrinol. 1976;
70:491-499), or had been transplanted to nude mice (Petersen et

10   al., J. Clin. Invest. 1984; 74:1358-1365). Basic studies on the
activity of human sebocytes and their regulation were made
possible only in the recent decade, as vital human sebaceous
glands were isolated (Kealex et al. Br. J. Dermatol. 1986;
114:181-188) and a culture model for human sebocytes could be

15   established in vitro (Xia et al., J. Invest. Dermatol. 1989;
93:315-321).

By means of modifications of the culture technique of Xia et
al. (1989), improvements have been achieved during the recent

20   years in view of reproducibility of the cultivation of human
sebocytes in vitro. Thus, Zouboulis et al. (Skin. Pharmacol.
1991; 4:74-83) omitted hydrocortisol in the culture medium by
means of adding human serum. Lee (in Epithelia: Advances in
Cell Physiology and Cell Culture; C.J. Jones, editors: Kluwer,

25   Dordrecht, 1990; 333-350) treated sebaceous glands with
collagenase before cultivating them in serum-free medium which
was enriched with additives. Also, primary sebocyte cultures
were obtained by omitting the 3T3 fibroblast cell layer which
served as an adherence base layer (Akamatsu et al., J. Invest.

30   Dermatol. 1992; 99: 509-511). Secondary cultures were kept in a
medium which was supplemented by lipid free serum (Zouboulis et
al., J. Invest. Dermatol. 1993; 101:628-633), and in serum-free
ceratinocyte containing basal medium without additives (Akamazu
et al., J. Invest. Dermatol. 1992; 99:509-511). In addition, it

35   was shown that the keratinocyte growth factor (KGF) remarkably
increases the yield and the proliferation of human sebocytes
(Chen et al., J. Invest. Dermatol. 1998; 110:84-89).

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In spite of these technical improvements, further progress is
strongly hampered by the situation that a cultivation of a
large number of sebocytes from isolated human sebaceous glands
5   is difficult. In particular, there is the difficulty to keep
the cell material in culture for a long period of time. As the
reason therefore, it is assumed that the sebocytes tend to
differentiate and to die via spontaneous cell membrane rupture
and the subsequent release of their content. The best result
10   yet achieved was that of Fujie et al. (Arch. Dermatol. Res.
1996; 288:703-708), who isolated sebaceous glands on the basis
of the technique of Xia et al. (1989) and cultivated sebocytes
by means of the method of dispersed cell culture through six
sub cultures in serum-free, keratinocyte growth medium without
15   a cell adherence layer.

Summary of the invention

It is an object of the present invention to provide sebocytes
20   (sebaceous cells) which can be maintained in culture through
higher number of subcultures. In this context, the provided
sebocytes shall approach, in the appearance of their
morphological, phenotypic and functional characteristics, those
of viable, normal human sebocytes, at least to such an extent
25   that they are suitable as cellular material or a cell culture
model for lipid containing, sebum-producing cells, and in
particular for sebocytes, for physiological, pathophysiological
and pharmaceutical evaluations and studies.

30   The object is solved by the provision of sebocytes which are
immortalised. Suitably, the cells of the present invention
derive from humans, because this is of primary interest for
useful applications. Sebocytes of this kind are present in the
human sebaceous gland cell line SZ95, which have been deposited
35   with the German collection of microorganisms and cell cultures
(DSMZ) under the depository No. DSM ACC2383.

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Description of the preferred embodiments

The present invention will, in the following, be described in
more detail with reference to the drawings (Figs.). Fig. 1 and
5   Fig. 2 show that immortalised sebocytes SZ95 provided by the
invention have maintained the epithelial, polymorphous
appearance of primary, normal sebocytes, from which they derive
(in the present case: from humans). In addition, the provided
immortalised sebocytes and the clones thereof (a clone means
10   cells which surely derive from a single cell) express the
characteristic 94-kD-large SV-40 large T-antigen, with which
coding DNA sequence transfection had been carried out, even in
the later subcultures. Fig. 1 shows (a) normal, human sebocytes
of the second subculture, from which the provided immortalised
15   sebocytes derive, (b) an adherent sebocyte culture as provided
by immortalised secocytes from the primary sub culture, and (c)
provided immortalised sebocytes (50th subculture of a clone).
All cells exhibit a similar ephithelial, polymorphous
structure. Fig. 2 shows cyto-centrifuged samples of (a)
20   provided immortalised sebocytes, and (b) of endothelial cell
culture cells HMEC-1 which served as a positive control, both
having been labeled with a monoclonal antibody against human
SV-40 large T-antigen. Both samples are labeled positively and
show that the human SV-40 large T-antigen is present
25   predominantly in the cell nucleus, partly also in the cytoplasm
of the cell. In (c), the expression of human SV-40 large T-
antigen in the provided immortalised sebocytes is demonstrated
by means of Western blot analysis. While human SV-40 large T-
antigen was not detectable in the non-transfected, normal human
30   sebocytes (lane 1) and in normal human ephitelial keratinocytes
(lane 2), the characteristic 94-kD large protein was determined
in protein extracts of the provided immortalised sebocytes in
the 34th subculture (lane 3) as well as in three isolated
clones (lanes 4, 5 and 6).
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The immortalised sebocytes of the present invention are
preferably of human origin. The meaning of the expression

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„sebocytes" is to be understood in the broadest sense, i.e.
relating to all cells which are, more or less, grease- or
lipid-containing and sebum-producing. Sebum is merely composed
of various fatty or lipid substances. In this connection, the
5   fat or lipid content of the cells may vary in terms of the
lipid substance fraction as well as in terms of content of the
lipid substance fractions. As a rule, but not necessarily, the
fatty or lipid substance content of the cells comprise free
fatty acids, triglycerides, wax, squalene, free cholesterol,
10   cholesterol esters, dihydroxy cholsterol and other steroids as
well as hydrocarbons. In particular, those immortalised
sebocytes are preferred which derive from human sebaceous gland
cells. A particularly good suitability for medicinal purposes
is achieved, if the sebocytes derive from human sebaceous gland
15   cells of the face.

An essential characteristic of the sebocytes of the invention
is their immortalization. Immortalised within the meaning of
the present invention basically means maintaining the vital
20   condition of the cells through multiple subcultures. The
immortalised sebocytes SZ95 of the present invention could be
maintained in culture, in the past observation time of about 4½
years, over more than 50 subcultures, whereas normal human
sebocytes can only grow up to three to six subcultures before
25   they die.

The immortalised sebocytes according to the present invention
can be obtained by means of transfecting normal sebocytes-
using, in the preferred embodiment, those of human origin and
30   particularly of the human sebaceous glands - with a DNA which
act on forming stable, inactive complexes with proliferation
inhibiting genes. A particularly successful immortalisation was
achieved in the present invention by transfecting normal human
sebocytes, in particular those derived from sebaceous glands of
35   the face, with a DNA comprising DNA sequences which encode for
the large T-protein of SV-40. The immortalising effect of the
SV-40 large T-antigen (protein) as well as the corresponding

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use of the coding DNA sequence for transfecting human cells is
basically known. Thus, immortalised cell lines had been
obtained through transfection with a DNA encoding for SV-40 T,
for example with other cells of epithelial origin (see Tohyama

5   et al., Tohoku. J. Exp. Med. 1997; 182:75-82; Bae et al.
Prostate 1998; 34:275-282), as well as of endothelial origin
(see Ades et al., J. Invest. Dermatol. 1992; 99:683-690 and WO-
A-92/17569).

10   It was found that by transfecting sebocytes with a gene
transfer method by means of applying cationic lipids
(LIPOFECTIN reagent, which is an 1:1 (w/w) liposomal
formulation containing the cationic lipids DOTMA [1,2-Diolyloxy
propyl-3-trimethyl ammonium chlorid] and DOPE

15   [Diolylphosphatidylehtanolamin] in membrane filtered water [1
mg/ml]), in which foreign DNA is taken up through endocytosis
into the cells via cationic lipid/DNA complexes (see Wang et
al., In. Vitro. Cell. Dev. Biol. 1991; 27A:63-74; Staedel et
al., J. Invest. Dermatol. 1994; 102:768-772), good results were

20   achieved for the immortalisation, while the transfection
mixture preferably contained in addition 0.25 to 2.0 vol.-% and
particularly 2.0 vol.-% LIPOFECTIN reagent as well as 0.05 to
0.5 wt.-% and particularly 0.5 wt.-% foreign DNA in a suitable
transfection buffer. The foreign DNA, like the one coding for

25   the SV-40 large T-protein, is typically inserted in a suitable
vector, by which the expression of the SV-40 large-T-protein is
enhanced, preferably by means of promotor and enhancer
sequences. If normal human sebocytes are transfected, in the
preferred embodiment, by the DNA which encodes for the SV-40

30   large T-antigen, it is to be expected that the provided
sebocytes express the large T-antigen of SV-40 after a
successful transfection and immortalisation. This was confirmed
for the immortalised sebocytes provided according to the
present invention by immuno-cytochemical means and by means of

35   Western blot analysis, using monoclonal antibodies against the
SV-40 large T-antigen.

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The thus obtained, immortalised sebocytes are preferably in the state of a cell line which, in this form, can be excellently used for the application purposes.

5   The immortalised sebocytes according to the present invention did grow, after their adaptation to serum-free culture medium, better than non-transfected, normal human sebocytes, and they maintained the capability of synthesising sebaceous gland specific lipids - contrary to the non-transfected normal human

10   sebocytes which had been maintained in serum-free medium. The immortalised sebocytes according to the present invention can thus serve as a continuously renewable and propagating cell line and can grow in defined culture media.

15   A particular value of the immortalised sebocytes according to the present invention is that they have features of non-transfected, normal and differentiated sebocytes in morphological, phenotypic and functional respects. Therefore, the immortalised sebocytes according to the present invention

20   can be excellently offered as models for physiological, pathophysiological and pharmacological studies. At the same time, the disadvantage of limited viability of conventional, cultured normal sebocytes of human origin is avoided. Accordingly, it was confirmed that the immortalised sebocytes

25   according to the present invention can substantially maintain the phenotype of normal sebocytes and can behave like non-transfected normal human sebocytes of the face in functional respects.

30   It was found that the immortalised sebocytes or the sebocyte cell line according to the present invention exhibit a polymorphous, epithelial appearance which is similar to that of non-transfected, normal human sebocytes. In cell culture, cells did grow in various sizes and intracellular structures, which

35   is indicative for various phases in cell maturation. Thus, cells of various sizes, in the average up to the 5-fold or 6-fold size with confluent growth, had been observed, which

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essentially corresponds to the cell growth increase of non-
transfected, normal human sebocytes with progressive
differentiation in vitro (in the average 4-fold to 5.5-fold
size difference). Furthermore, the immortalised sebocytes
5   according to the present invention was found to be rich in
fatty substance or lipid particles in the cytoplasm, like in
non-transfected, normal human sebocytes. The synthesis of the
characteristic sebaceous gland lipids squalene and wax esters,
which are common for normal human sebocytes, was confirmed
10 experimentally in the course of the present invention.
Furthermore, the immortalised sebocytes of the present
invention synthesised free fatty acids, which, again,
correlates with the findings of non-transfected, normal human
sebocytes in vitro - and even after a high number of sub-
15 cultures.

Also, expression markers which confirm a sebocyte origin and
which indicate a viable differentiation, were confirmed as
typical indications for sebocytes of the immortalised sebocytes
20 or sebocyte cell line of the present invention. Thus, the
immortalised sebocytes or the sebocyte cell line expressed
antigens which are typical for the human polymorphous
epithelial mucous protein group, such as the sebaceous gland
antigen, the human milk fat globulins 1 and 2, the human
25 epithelial sialomucine, the Thomsen-Friedenreich antigen, the
mucin-like carcinogen associated antigen and the epithelial
membrane antigen. This was confirmed in the course of the
present invention by immuno cytochemical means and by means of
Western blot analysis. In addition, the immortalised sebocytes
30 or the sebocyte cell line of the present invention expressed
keratinic antigens typical for non-transfected, normal human
sebocytes, such as those of sub-classes 7, 13 and 19. The
antigen phenotype thus demonstrated the sebocyte origin as well
as the differentiation of the sebocytes.

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Also in functional terms, the immortalised sebocytes or the
sebocyte cell line of the present invention are similar to non-

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transfected, normal human sebocytes. Thus, the immortalised sebocytes of the present invention responded to the effects of androgens, such as e.g. by 5α-dihydro testoterone, by enhancing their in vitro proliferation. In addition, the

5   immortalised sebocytes or the sebocyte cell line of the invention possessed the capability of varying their proliferation by the effects of retinoids, in particular those of the non-aromatic type (e.g. 13-*cis*-retinoic acid, all-*trans*-retinoic acid).

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A preferred embodiment of the present invention is that the immortalised and preferably the human sebocytes are cloned. This is advantageous because the immortalised sebocytes or the thus generated sebocyte cell line are well defined and

15   specifically characterized by means of their unique genomic basis. A cloned and immortalised human sebocyte cell line was suitably obtained by gradually diluting immortalised sebocytes in culture vessels long enough, until the cell division started again from only one cell per culture vessel. This could be

20   observed and controlled by means of microscopic observations.

Accordingly, it was found in the course of the present invention that the obtained immortalised human sebocytes or the thus obtained sebocyte cell line essentially maintained the

25   sebocyte identity compared to non-transfected, normal human sebocytes. This was confirmed by characteristic determinations and functional tests.

A sebocyte cell line with the specification SZ95, which entails

30   the above-mentioned advantages of the present invention, is represented by the sebaceous gland cell line which was deposited with the DSMZ under the depository No. ACC2383.

Accordingly, the immortalised sebocytes or the sebocyte cell

35   line according to the present invention offer excellent possibilities for useful applications. In general, the sebocytes or the sebocyte cell line according to the invention

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can be used for diagnostic, for therapeutic, or for cosmetic
uses. Specifically, the sebocytes or the sebocyte cell line
described above may serve for developments and studies of the
physiology or the pathophysiology of lipid-containing, sebum

5   producing cells, in particular of human or animal sebaceous
gland cells, as well as their role in pathophysiologic
processes of the skin and in skin diseases like, e.g., acne.
With the help of the invention the generation of acne and/or
seborrhoe and/or other diseases, especially of skin diseases in

10   which the sebaceous gland function plays a role or may play a
roll, can be studied. The products of the present invention
further serve as excellent models for testing and for
evaluating anti-acne compounds and/or anti-seborrhoe compounds
or therapeutic agents, but also therapeutic agents against

15   diseases, especially skin diseases, in which the sebaceous
gland function plays a role or may play a role. Especially for
the performance of clinical studies, such in vitro studies on
pharmacological properties of medicaments are useful.

20   The sebocytes or the sebocyte cell line of the invention as
described above additionally have the advantage that further
cell culture systems may be established. This includes the
development of simple and of complex cell culture systems.
Simple cell culture systems means, as a rule, two-dimensional

25   one-layer or multi-layer adherent cultures or non-adherent
cultures and are, for example, formed by means of addition of
the above-described sebocytes to other cell types, or by means
of cultivating them through semi- or non-permeable membranes
(Schwartz et al., J. Surg. Res. 1998; 76: 79-85; Nackman et al.

30   Surgery. 1998; 124: 353-361). Complex cell culture system
means, as a rule, a three-dimensional cultivation of one-layer
or multiple-layer cultures and are, for example, formed by
cultivating the cells as spheroids, on spheroids, in collagen
or in other jelly materials or in an artificial skin-like

35   structure (Korff and Augustin, J. Cell. Biol. 1998; 143:1341-
1352; Hamamoto et al.; J. Biochem. (Tokyo) 1998; 24:972-979;
Desoize et al. Anticancer. Res. 1998; 18.4147-4158; Hamilton,

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Cancer. Lett. 1998; 131:29-34; Niemann et al., J. Cell. Biol.
1998; 143:533-545; Awata et al., J. Gastroenterol. Hepatol.
1998; 13Suppl:S55-61; Voura et al., Microsc. Res. Tech. 1998;
43:265-275; Pipili-Synetos et al., Br. J. Pharmacol. 1998;

5   125:1252-1257; Vasile et al., J. Histochem. Cytochem. 1999;
47:159-168; Michalopoulos et al. Hepatology. 1999; 29:90-100;
Trent and Kirsner., Int. J. Clin. Pract. 1998; 52:408-413;
Fransson et al., Br. J. Dermatol. 1998; 139:598-604;
Konstantinova et al., Arch. Dermatol. Res. 1998; 290:610-614;

10   Black et al., FASEB J. 1998; 12.1331-1340; Zhao et al.,
Biochem. Biophys. Res. Commun. 1999; 254:49-53).

A particularly useful application relates to the generation of
three-dimensional cell assemblies, or constructions or

15   reconstructions of organ-like structures based on the sebocytes
or the sebocyte cell line of the invention. For this purpose,
the sebocytes are used alone, but preferably in addition to
further skin generating cells, in particular with
keratinocytes, fibroblasts, melanocytes, endothelial cells,

20   Langerhans' cells and/or cells from the hair follicle. For the
generation of three-dimensional cell assemblies, or
constructions or reconstructions of organ-like structures, a
support scaffold with collagen or other jelly materials and/or
with parts of inactivated tissue is provided first, and then

25   the aforementioned cells are applied in or onto this support
scaffold. This method is basically known to the man skilled in
the art, and samples are commercially available (Trent and
Kirsner, Int. J. Clin. Pract. 1998; 52:408-413; Fransson et
al., Br. J. Dermatol. 1998; 139:589-604; Konstantinova et al.,

30   Arch. Dermatol. Res. 1998; 290:610-614; Black et al., FASEB. J.
1998; 12:1331-1340; Zhao et al., Biochem. Biophys. Res. Commun.
1999; 254:49-53). An „artificial skin" or a skin substitute is
produced thereby, which offers excellent possibilities for the
transplant or grafting medicine, for the reconstruction of

35   damaged skin portions such as, e.g., burnt skin, or for the
therapy of skin lesions. With the help of the present
invention, such an „artificial skin" can synthesise

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lipids/sebum in sufficient amounts, when the sebocytes of the
present invention are incorporated into the constructions.

A further field of useful applications relates to the
5   manufacture of products which derive from the sebocytes or the
sebocyte cell line of the invention. This includes the
isolation and purification of cellular substances, such as
lipids, proteins, DNA and/or RNA. Since the cells are
immortalised, they are maintained to be offered as a continuous
10   source for such cellular substances. Specific examples for very
suitable substances, which can be obtained accordingly from
these cells, include: skin lipids for their use in topical
agents and medicaments, the antigenic proteins which are
mentioned in Example 3 below in connection with a phenotypic
15   characterisation of sebocytes, and, further, the generation of
plasmid DNA or vector DNA. The generation of plasmid DNA or
vector DNA is carried out by means of genetic engineering known
to those skilled in the art. Accordingly, genes can be
retrieved which induce lipid production. Especially with such
20   suitable plasmid and vector constructions, which also includes
the generation of viral vectors, again other cells or organisms
can be modified or transfected.

The present invention will be explained in more detailed by
25   reference to the following, non-limiting examples.

Examples

For the examples described below, the following embodiments as
30   to the materials and methods may be used. The examples should
not be interpreted as being limiting.

Cell Cultures

35   If not indicated otherwise, all cells were maintained in a
medium as adherent cultures which consisted of a modified
DMEM/HAM's F12-Medium (1:1) (available from Biochrom, Berlin,

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Germany) having 2 mM N-Acetyl-L-alanyl-L-glutamine which was
supplemented with 10% heat-inactivated, fetal calf serium (FCS;
Biochrom) as well as 50 μm/ml gentamycin (available from Gibco-
BRL, Karlsruhe, Germany). The culture was maintained in a humid
5   atmosphere containing 5% CO_2 at 37°C and the culture medium was
renewed every 2 to 3 days.

Isolation and Cultivation of normal human sebocytes

10   Normal sebocytes were isolated from the facial skin of a 87-
year old female patient undergoing surgery, as reported by Xia
et al. (J. Invest. Dermatol. 1989; 93:315- 321). The isolated
sebaceous glands were cultured without feeder layer in the
standard medium supplemented with 9 ng/ml epidermal growth
15   factor (EGF), 9 ng/ml keratinocyte growth factor (KGF) (both
available from Boehringer Mannheim, Germany), 0.4 μg/ml
hydrocortisone (available from Sigma, Deisenhofen, Germany) as
well as 10^{-9} M cholera toxin (available from Calbiochem, Bad
Soden, Germany). Primary normal sebocyte cultures resulted as
20   outgrowths from the periphery of the sebaceous gland lobules.

Immunocytochemical Tests

Dispersed cells of sub-confluent normal sebocyte cultures were
25   attached to glass slides by cytocentrifugation. The samples
were air dried and fixed with cold acetone for 10 minutes. The
preparations were subsequently incubated with the respective
monoclonal antibody or a control antibody at room temperature
for 30 minutes. Bound antibodies were detected by coupling with
30   a 1:100 dilution of a monoclonal antibody conjugate from
rabbit/anti-mouse IgG (H+L) and an alkaline phosphatase/anti-
alkaline phosphatase complex (available from Dianova, Hamburg,
Germany) at room temperature for 30 minutes. Primary and
secondary monoclonal antibodies were diluted in solutions
35   containing 10% RPMI-1640 and 10% FCS at a pH of 7.4. The
washing steps were conducted three times with PBS buffer

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without Ca^{2+} and Mg^{2+} (available from Biochrom). The
preparations were stained for 30 minutes in buffered solution
(pH 8.8) with Neufuchsin as an adherent agent and a naphthol
salt as a coupling agent (both from Sigma), counter-stained
5   with Mayer's Haemalum (Merck, Darmstadt, Germany), covered and
judged with a light microscopy.

Isolation and Quantitation of proteins

10   Cell cultures were washed twice with PBS, lysed directly in the
culture dishes by a cold solution which consisted of 50 mM
HEPES, 1% Nonidet P-40 (available from ICN, Aurora, OH, USA),
150 mM NaCl as well as a protease inhibitor (Complete^{TM} Mini;
available from Boehringer Mannheim), subsequently scrubbed and
15   harvested in small centrifugation dishes to isolate cellular
proteins. The obtained material was homogenized by ultrasonic
disruption, subjected to centrifugation and the supernatants
were held on ice. Bicinchoninic acid (BCA-protein assay;
available from Pierce, Rochford, IL, USA) was added to
20   visualize the total protein and the protein concentration was
quantitated by measurement of absorption at 550 nm.

Western blot analysis

25   Aliquots of the isolated proteins (20 µg) were heated to 95°C
for 15 minutes. One-dimensional SDS/PAGE electrophoresis was
conducted with each sample on 7.5% gels. Then, proteins were
transferred to a transfer membrane (Immobilon-P of PVDF;
available from Millipore, Eschborn, Germany) utilizing a
30   standard blot system (available from Bio-Rad, München,
Germany). The blots were incubated with primary monoclonal
antibody at room temperature for 60 minutes and subsequently
with horse radish peroxidase-conjugated goat/anti-mouse
monoclonal antibody and goat/anti-rabbit monoclonal antibody,
35   respectively (available from Oncogene Science) in a dilution of
0.2 µg/ml at room temperature for 60 minutes. After thorough

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washing, the signals were visualized by a chemiluminescence
method utilizing a standard assay (ECL, available from
Amersham, Braunschweig) on X-ray sensitive films (XAR 5;
available from Kodak, Rochester, NY, USA), whereby various
5   illumination intervals were adjusted.

Oil Red and Nile Red Staining

Cells grown in chamber slides were incubated either with 0.6%
10   Oil Red (Sigma) in 60% isopropanol for 15 to 120 minutes or
with 1 µg/ml Nile Red dye (available from Kodak) for 15 minutes
at room temperature, as reported by Xia et al. (1989) (supra).
The cultures were then observed under a light microscope (after
Oil Red stain) or a fluorescence microscope using a 450 to 500
15   nm bandpass excitation filer by light emission of > 580 nm ·
(after Nile Red stain).

Flow cytometry

20   Dispersed, non-labeled cells were determined for their cell
size using a conventional sorter, while cells labeled with Nile
Red dye (available from Kodak) were assessed for lipid content
by flow cytometry on a fluorescence basis. 10.000 cells per
sample were tested.
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Labeling and extraction of lipids

Cell cultures were maintained in culture medium for 2 days and
then radioactively pulsed via the sodium salt of $[2-^{14} C]$
30   acetic acid (45 - 60 mCi/mmol; available from DuPont-NEN,
Boston, MA, USA) with a concentration of 0.5 µCi/ml in RPMI-
1640 medium supplemented with 2 mM L-glutamine, 10% heat-
inactivated FCS and 100 IU/ml penicilline and 100 µg/ml
streptomycine. Incubation was continued for further 24 hours.
35   Lipids were isolated from cultured cells and from the
supernatant culture medium and separated into neutral lipids,

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fatty acids and phospholipids (see Seifert et al. J. Invest.
Dermatol. 1997; 108: 375).

5 The size separation into fractions and the visualization of
neutral lipids and free fatty acids was obtained by high
performance thin layer chromatography (HPTLC) conducted on 20 x
10 cm² silica gel-coated glass plates (available from Merck,
Darmstadt, Germany). The plates were pretreated with n-hexane
and dried for 24 hours. The samples were applied by an
10 automatic lipid applicator (Linomat IV; Camag, Berlin,
Germany). Chromatograms of the neutral lipids were developed in
a n-hexane-diethylether solution (9:1) on 9 cm, dried and post-
developed in a solution of chloroform/diethylether,
ethylacetate (80:4:16) on 4.5 cm. For illumination,
15 illumination sheets (TR 2040S, available from Fuji, Tokyo,
Japan) were used which were scanned using an image analyser
("BAS 1000 Bio-Imaging Analyser", Fuji). Lipid standards were
used as comparative samples.

20 Growth behavior tests

Cells were seeded in 96 well culture plates at densities of 0.5
to 4 x 10³ cell/well. Cell proliferation was assessed by the 4-
methylumbelliferylheptanoatefluorescence assay and measured
25 automatically (Zouboulis et al. Melanoma. Res. 1991; 1:91-95).

To this extent, on the day of evaluation, the culture medium
was removed, the cells were washed twice with PBS and 100 μl of
a 100 μg/ml solution of 4-methylumbelliferyl heptanoate (Sigma,
30 Deisenhofen, Germany) in PBS were added to each well. The
plates were then incubated at 37°C for 30 minutes and released
fluorescence was measured by a suitable fluorescence measuring
device (Titertec-Fluoroscan II; Flow, Meckenheim, Germany).
Fluorescence units were obtained at 355 nm excitation and 460
35 nm emission filters.

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Treatment with 5α-dihydrotestosterone and retinoids

5α-dihydrotestosterone (5α-DHT; Sigma) was dissolved in DMSO
and subsequently in serum-free, phenol-free modified DMEM/Ham's

5   F12-Medium (1:1) (Gibco-BRL) with 2 mM n-Acetyl-L-alanyl-L-
glutamine which was supplemented with 5 ng/ml EGF, 50 µg/ml
bovine pituary extract, 1 mg/ml fatty acid-free bovine serum
albumine (Boehringer Mannheim) and 50 µg/ml gentamycine to
obtain a final concentration of 10^{-6} M 5α-DHT and 0,1% DMSO.

10 0,1% DMSO alone served as a control. The cells (0.5 to 2 x
10^3/well) were treated with 5α-DHT for 18 days.

For the treatment with retinoids, all-*trans*-retinoic acid, 13-
cis-retinoic acid and acitretin were dissolved in DMSO and

15 subsequently placed in serum-free modified DME-Medium/Ham's
F12-Medium (1:1) with 2 nM N-Acetyl-L-alanyl-L-glutamine which
was supplemented with 5 ng/ml EGF, 50 µg/ml bovine pituary
extract, 1 mg/ml fatty acid-free bovine serum albumine and 50
µg/ml gentamycine to obtain a final concentration of 10^{-7} M

20 retinoid and 0,1% DMSO. 0,1% DMSO alone served as a control.
Retinoids were handled under dim amber light. The cells (0.5 to
1 x 10^3/well) were treated with the retinoids for 9 days.

Statistical Analysis

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Growth studies were assessed in sextuplicate formulations of 96
well plates. All other experiments were performed in triplicate
formulations.

30 Example 1

Transfection of normal human sebocytes

The vector used for the transfection of normal human sebocyte
35 designated pSVT was a plasmid construct on the basis of PBR322

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comprising sequences for the SV-40 large T protein where its
protein expression was driven by the Rous Sarcoma Virus long-
terminal repeat (see Dutt et al. Oncogene 1990; 5:195-200; Wang
et al. In. Vitro. Cell. Dev. Biol. 1991; 27A:63-74). Human

5   sebocyte cultures in the second subculture were grown to 50%
confluency in 35 mm culture dishes (Becton Dickinson, Plymouth,
UK) and used for transfection. The transfection was performed
on the basis of a gene transfer method using cationic lipids.
To this extent, the LIPOFECTIN reagent was utilized which

10   contained a 1:1 (w/w) liposomal formulation of cationic lipids
DOTMA (1,2-Diolyloxypropyl-3-trimethylammoniumchloride) and
DOPE (Diolylphosphatidylethanolamine) in membrane filtered
water (1 mg/ml). To this extent, the culture medium was
removed, the culture cells were washed twice with serum-free

15   medium (Opti-MEM from Gibco-BRL) and incubated in this medium
for 4 hours. The medium was then replaced by a transfection
mixture consisting of an antibiotic-free amount of Opti-MEM
medium (1.5 ml) with a suitable amount of the LIPOFECTIN
reagent (Gibco-BRL; 5 - 30 µl, most preferably 1.5 vol-%) as

20   well as a suitable amount of pSVT DNA (1-10 µg) in a solution
having 0.5 ml PBS (final DNA concentration, most preferably 0.5
wt.-%). The cultures were incubated in humid atmosphere
containing 5% CO_2 at 37°C for 24 hours. The cultures were
finally washed twice with culture medium and further maintained

25   in sebocyte culture medium as described above.

After the transfection treatment, a drastically diminished
viability of the pSVT-treated sebocytes was observed during 4
weeks. Particularly with the use of optimal amounts of

30   LIPOFECTIN reagent and pSVT DNA proliferating sebocyte colonies
occurred. These cells (SZ95) were able to be passaged to date
more than 50 times. They are still viable upon the observation
period of 4.5 years.

35   Example 2

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Cloning of immortalized human sebocytes

The thus immortalized human sebocytes SZ95 were seeded in 96
well culture plates using a dilution series with geometrically
5   descending cell numbers of 1×10^2 cells in the first series
until theoretically zero cells were reached in the last series
(Zouboulis et al. in "The malignous melanome of the skin", C.E.
Orfanos and C. Garbe (eds.) Zuckschwerdt, München, Germany:
1990; 158-168). The cells were maintained in standard culture
10 medium supplemented with 5 mg/ml EGF and 3 ng/ml KGF. Growing
cells were regarded as clones in case they were derived from a
single cell per well which was observable by light microscopic
experiments. Thus, cloned SZ95 cells were obtained.

15 ## Example 3

Characterization of the immortalized human sebocytes

Detection of SV-40 large T antigene

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The expression of SV-40 large T antigene in immortalized
sebocytes was detected immunocytochemically and by Western blot
analysis using a monoclonal anti-human SV-40 large T antigene
antibody from mouse serum (Oncogene Science, Cambridge, MA,
25 USA) which was diluted for immunocytochemical analysis to
1:1000 and for Western blot analysis to 1:100. Human normal
epidermal keratinocytes, dermal fibroplasts and as a positive
control endothelial cells HMEC-1 immortalized by SV-40 large T
antigene (see WO-A-992/175 69) were used as a comparison.
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The immunocytochemical experiment of the immortalized sebocytes
according to Example 1 with the monoclonal antibody against SV-
40 large T antigene resulted in a strong, mostly nucleic,
partly cytoplasmatic staining (see Fig. 2a). Normal
35 keratinocytes and fibroplasts were uniformly negative vis-à-vis
the SV-40 large T protein and the HMEC-1 cells as a positive

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control showed mostly a nucleic, partly cytoplasmatic staining
for the SV-40 large T protein (see Fig. 2b).

Fig. 2c shows the results of Western blot analysis of SV-40
5   large T antigene expression in non-transfected normal human
sebocytes (lane 1), in normal human epidermal keratinocytes
(lane 2), in immortalized sebocytes according to the present
invention (34th sub-culture; lane 3) as well as in various
cloned sebocytes according to the present invention (lanes 4, 5
10 and 6). A band at 94 kD was confirmed to be the immortalized
sebocyte line as well as its clones which confirmed the
expression of the SV-40 large T protein (see Harlow et al. J.
Virol. 1981; 39:861-869).

15 <u>Phenotypic characterization of the immortalized sebocytes</u>
<u>according to the present invention</u>

The morphology of the immortalized sebocytes SZ95 according to
Example 1 was epithelial and exhibited a polymorphous
20 appearance with cells of different size, whereas numerous
droplets could be observed in the cytoplasm (see Fig. 1b and
1c).

Immunocytochemical experiments of the immortalized sebocytes
25 according to the present invention with respective antibodies
resulted in a positive finding against the sebaceous gland
antigene in contrast to normal epidermal keratinocytes which
were not stained by the monoclonal antibody against the
sebaceous gland antigene (see Fig. 3). Fig. 3 shows the
30 immunocytochemical results on cytocentrifugation preparations
(a) of the immortalized sebocytes according to the present
invention as well as (b) normal human epidermal keratinocytes.
The preparations were stained with an monoclonal antibody
against the sebaceous gland antigene. While the immortalized
35 sebocytes of the present invention exhibited a positive
cytoplasmatic staining, the normal human epidermal
keratinocytes were not stained.

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Moreover, the expression of the keratines 7, 13 and 19 as well
as various proteins of the human polymorphous epithelial mucine
group in the immortalized sebocytes was detected by Western

5   blot analysis, whereas human keratinocytes expressed only
keratine 13 (see Fig. 4). In Western blot analysis according to
Fig. 4, extracted total protein of immortalized sebocytes
according to the present invention (34th sub-culture; lane 1),
various cloned immortalized sebocytes according to the present

10   invention (lanes 2, 3 and 4) as well as normal human epidermal
keratinocytes (lane 5) were applied for identification of the
expression of human epithelial sialomucin (ESM) (> 400 kD),
human milk fat globuline-1 (HMFG-1) (400 kD), human milk fat
globuline-2 (HMFG-2) (80 - 400 kD), mucine-type carcinome-

15   associated antigene (MCA) (350 kD), epithelial membrane
antigene (EMA) (250 - 400 kD), Thomsen-Friedenreich antigene
(TF antigene) (155 kD), Keratin 7 (54 kD), Keratin 13 (54 kD),
Keratin 19 (40 kD) as well as 5α-reductase of type 1 (5α-
Red.1) (21 - 27 kD). The immortalized cell line and its clones

20   according to the present invention expressed all tested
proteins, whereas keratinocytes only expressed Keratin 13 and
5α-reductase of type 1.

Lipid Synthesis

25   The staining with Nile Red and the assessment by fluorescence
microscopy showed the presence of lipids in the cell cytoplasm.
In the immortalized sebocytes SZ95 of the present invention
(see Fig. 5), stained by Nile Red fluorescent dye directed to

30   neutral lipids resulted in individual or in grouped lipid
droplets which were optionally divided in the cytoplasm of the
sebocytes. The immortalized sebocytes according to the present
invention decreased their content from 510 fluorescent units
per cell (median value) in serum-containing medium to 429

35   fluorescent units per cell (median value; i.e. -16%) in serum-

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free medium detected by fluorescence cytometric experiments of
cells stained with Nile Red.

The immortalized sebocytes according to the present invention
5   of Example 1 synthesized various fractions of neutral lipids
including the typical sebaceous gland lipids squalene and wax
ester as well as triglycerides, cholesterol, cholesterol ester,
diglycerides, lanosterol and free fatty acids. This was
observed throughout 25 to 40 sub-cultures. The result in shown
10   in Fig. 6, where HPTLC-fractionized lipids were detected after
pulse recording of radioactively labeled sodium acetate by
means of radiometric image evaluation with two selected
immortalized and cloned sebocyte cultures (see lanes 3 and 4 as
well as 5 and 6, respectively). As shown by lanes 3, 4 and 5,
15   the cells synthesized multiple fractions of neutral lipids
including squalene (Sq), wax ester (WE) as well as
triglycerides (Tg), cholesterol (Cho), cholesterol ester (ChE),
diglycerides (Dg), lanosterol (Lan) as well as free fatty acids
(FFA). All neutral lipids were also found but in a lesser
20   extend in the extracellular supernatant (see lane 6). For
comparison in lane 1, lipid standards, in lane 2 human sebum,
and in lane 4, free fatty acids extracted from cells were
applied. As further comparison, lanes 7 and 8 showed the
results of keratinocytes, whereas lane 7 showed the presence
25   mainly of cholesterol and triglycerides in the cells, while
from the supernatant (lane 8), mostly cholesterine was found.

Proliferation studies

30   A logarithmic proliferation pattern of the immortalized
sebocytes according to the present invention was detected under
normal culture conditions with population doubling times of
52.4 ± 1.6 independent from the original culture cell
densities. To this extent, Fig. 7 shows the proliferation of an
35   immortalized cloned sebocyte cell line (SZ95) over 18 days in
sebocyte medium.

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The proliferation of the immortalized sebocytes was reduced
after addition of serum-free medium but was retrieved after
addition of 5α-DHT. This is shown in Fig. 8 for an exemplary
5   sebocyte clone where the proliferation of the cells (seeding of
2.000/well) was observed over 18 days in serum-free medium
(control) as well as in serum-free medium which was
supplemented with 10^{-6} M 5α-DHT. After the 8th day, 5α-DHT
increased the proliferation of the cells significantly which
10   was shown by the determined population doubling time of 136
hours (control) and 53.7 hours (5α-DHT-treated cells (*,
$p<0.05$; **, $p<0.01$).

The influence of retinoids on the immortalized cells showed a
15   differentiated response of the proliferation behavior. While
some clones showed inhibition of proliferation by retinoids
(typically distinctively pronounced in the order of 13-*cis*-
retinoic acid > all-*trans*-retinoic acid >> Acitretin), other
clones were stimulated in proliferation (for example by all-
20   *trans*-retinoic acid and 13-*cis*-retinoic acid) corresponding to
the proliferation response of normal human epidermal
keratinocytes. This is shown in Fig. 9 (*, $p<0.05$; **, $p<0.01$;
***, $p<0.001$).